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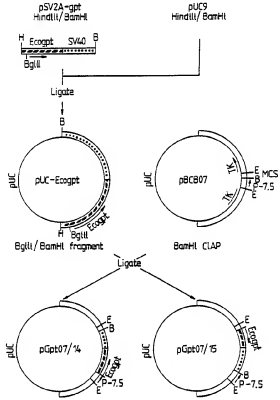
(57) Abstract

Recombinant fowlpox virus or related avian poxvirus is characterised by the inclusion of foreign DNA in the virus genome. The foreign DNA sequence may comprise the Ecogpt gene coupled with a second gene and be inserted into a non-essential region of the virus genome.



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"RECOMBINANT POXVIRUSES"

This invention relates to recombinant poxviruses and their construction, and in one particular aspect this invention relates to recombinant fowlpox virus and the use thereof as a vehicle for the expression of foreign genes particularly for the expression of avian disease antigens.

Poxviruses are large DNA viruses that replicate within the cytoplasm of infected cells. Vaccinia virus, the type species of the orthopox virus group, has been widely studied because of its role as the vaccine virus for smallpox in man. In recent years, recognition that the TK gene was a non-essential region of the vaccinia virus genome, followed by mapping and nucleotide sequence

determination, has made it possible to insert and express a wide variety of foreign genes through the medium of recombinant vaccinia viruses (1-12). Such recombinant vaccinia viruses have the potential to deliver vaccine antigens to a variety of animal species (13). However, the risk of spread to man and the potential for disease problems from the widespread use of vaccinia virus in animals makes the construction of recombinants based on host specific poxviruses desirable, e.g. fowlpox virus for poultry or Orf for sheep vaccines. To achieve this requires considerable understanding of the molecular biology of these viruses, including the identification of non-essential regions into which foreign DNA might be inserted.

In one aspect, the present invention, which now makes possible a range of recombinant avian vaccines, is based on the mapping and sequencing of a non-essential region of the FPV genome. This apparently non-essential region is the thymidine kinase (TK) gene, and it permits the introduction of foreign DNA into fowlpox virus (FPV) without impairing the potential for virus replication and the production of live infectious recombinant viruses.

According to this aspect, the present invention provides recombinant fowlpox virus related avian poxvirus characterised by the inclusion of foreign DNA in the virus genome.

In a preferred embodiment of this aspect, the foreign DNA is inserted into a non-essential region of the fowlpox virus genome, such as the TK gene or in virus DNA sequences controlling expression of the TK gene. In other embodiments, the foreign DNA may be inserted into intergenic regions of the

fowlpox virus genome, for example regions preceding the TK gene or after the TK gene in such a manner as not to disrupt its function or the function of genes immediately upstream or downstream of the TK gene. The foreign DNA may also be inserted between genes (intergenic) in essential regions of the fowlpox virus genome, or within genes or their regulatory sequences (intragenic) in other non-essential regions of the whole genome.

In a further aspect, the invention provides an avian disease vaccine comprising recombinant fowlpox virus wherein a foreign DNA sequence encoding an antigen characteristic of the said avian disease has been inserted into the TK gene of the fowlpox virus or in virus DNA sequences controlling expression of the TK gene.

By mapping and nucleotide sequence analysis (14,15) it has been shown that the FPV TK gene is characterised by an open reading frame of 183 codons commencing 279bp upstream of the central Xba 1 site of the nucleotide sequence as shown in Figure 8, and terminating 273bp downstream of this Xba 1 site. Confirmation of this sequence as the FPV TK gene is given by homologies with the vaccinia TK gene at nucleotide and amino acid levels, and the expression of TK enzyme which results from its insertion in TK^- vaccinia virus.

According to another aspect, this invention provides an avian disease vaccine comprising recombinant fowlpox virus wherein a foreign DNA sequence encoding an antigen characteristic of the said avian disease has been inserted in the region of the fowlpox virus commencing 279bp before and terminating 273bp after the central Xba 1 site of the

nucleotide sequence shown in Fig.8, or in sequences controlling expression of this sequence.

It is envisaged that in accordance with this invention vaccines will become available for protection against a wide variety of avian diseases such as those caused by viruses, bacteria, protozoa, metazoa, fungi and other pathogenic organisms, by insertion of DNA sequences encoding appropriate antigens characteristic of these diseases into the FPV genome as broadly outlined above.

A general method for the insertion of foreign DNA into vaccinia virus is based on the selective inactivation of the TK gene and uses 5-bromodeoxyuridine to select TK^- recombinant viruses (3). Since there are no TK^- avian cell lines available, selection of TK^- FPV recombinants is not possible. Other general methods for the construction of recombinant vaccinia viruses are known, and are discussed in detail hereinafter.Some of these approaches may be applicable to the insertion of foreign DNA into other poxviruses, however, none have yet been successfully applied to FPV. As described in detail below, it has now been demonstrated that the Escherichia coli xanthine-guanine phosphoribosyl transferase (Ecogpt) gene can be used as a dominant selectable marker for the insertion of foreign genes into poxviruses and that this technique has a number of advantages over other methods for the construction of recombinant viruses.

One of these is that the selection for the Ecogpt gene operates in a wide variety of cell types and therefore is potentially applicable as a dominant selectable marker for the insertion of foreign genes

into other poxviruses (29). Mycophenolic acid, the antibiotic which provides the basis for selection of the Ecogpt gene (together with the required presence of xanthine, hypoxanthine, aminopterine, and thymidine), is a potent inhibitor of fowlpox virus in chicken embryo skin (CES) cell cultures. This has allowed the Ecogpt gene to be used as a dominant selectable marker for the insertion of foreign DNA into the TK gene of FPV. Expression of foreign genes by vaccinia viruses requires their being positioned near unique vaccinia virus transcriptional signals (1-4, 7-9), and it has been shown that the FPV TK promoter is similar to vaccinia promoters and that it operates in vaccinia virus (14,15). Vaccinia virus promoters have now been used to express foreign antigen coding sequences in FPV.

This invention therefore also offers methods of constructing recombinant fowlpox viruses or related avian poxviruses, which methods are characterised by the introduction of foreign DNA into the TK gene of the virus or into virus DNA sequences controlling expression of the TK gene.

At this stage the vaccinia virus promoter sequences P7.5 and PL11 have been used to express foreign genes in FPV. Recombinant FPVs have been constructed which contain the Ecogpt gene (demonstrated by hybridization) and which express the Ecogpt gene since they are able to grow in MXHAT (mycophenolic acid, xanthine, hypoxanthine, aminopterine, thymidine) selective conditions. This gene is under the control of the P7.5 promoter which clearly demonstrates that this and possibly other vaccinia virus promoters can be used to express foreign genes in FPV. The PL11 promoter expresses

the influenza HA gene. The level of expression by this promoter may be modulated in these recombinants since the translational initiation codon of the 11kd protein gene is present as well as the inserted HA gene initiation codon.

The insertion of the Ecogpt and HA genes into FPV and the recovery of viable virus able to grow in tissue culture clearly demonstrates that the FPV TK gene is not essential for FPV growth in vitro. This establishes the FPV TK gene as a non-essential region into which foreign DNA can be inserted and expressed. Further it provides the means to construct TK^- FPVs which by analogy with TK^- vaccinia viruses (32) would be expected to have reduced virulence in poultry and thus be useful as vaccines against FPV themselves.

Using the techniques described herein, two foreign genes have been inserted into FPV (the Ecogpt gene and the influenza HA gene). Vaccinia virus recombinants carrying three vaccine antigens have been constructed and shown to simultaneously induce antibody responses to all three vaccine antigens when inoculated into animals (33). Additional poxvirus promoters and vaccine antigen genes in tandem with the Ecogpt can be inserted within the FPV TK gene in the general insertion vector pDB22 described herein. In this manner, recombinant FPVs can be constructed which will vaccinate poultry simultaneously against two, three or more diseases. Recombinant FPVs carrying multiple vaccine antigens would be a very cost effective way to deliver vaccines to poultry.

Included in the recombinant fowlpox viruses of this invention are thymidine kinase negative fowlpox viruses, which in their own right would be

vaccines against fowlpox. In such viruses, TK production would be inactivated by the introduction of foreign DNA into the TK gene or controlling sequences, or by the excision of DNA essential for TK gene expression. By analogy with TK^- vaccinia and herpes viruses, TK^- fowlpox viruses would have reduced virulence and pathogenic potential in poultry and other avian species, and correspondingly enhanced usefulness as fowlpox vaccines and vehicles for the delivery of other vaccine antigens.

It is to be understood that the utility of recombinant fowlpox viruses according to this invention is not limited to the delivery of antigens. It is envisaged that they might also be useful for the delivery of a variety of other proteinaceous materials, e.g. growth hormones or immunomodulators, capable of being produced by expression of an appropriate gene.

As mentioned above, in recent years vaccinia virus has been the subject of interest as a vehicle for the expression of various foreign genes and thus as a basis for recombinant vaccines. A variety of techniques have been developed for the construction of recombinant poxviruses based on vaccinia virus. In general, they rely on homologous recombination between vaccinia virus flanking sequences bounding the foreign gene of interest and the virus genome during simultaneous infection of cells with virus and transfection with recombinant plasmid. From the progeny virus, recombinant viruses representing less than 0.1% or 0.01% of the population have to be selected and plaque purified. This is achieved by plaque hybridization with an appropriate radiolabelled probe; or by selection for

TK^- recombinants when the foreign gene has been inserted into the TK gene of the virus; or by the co-expression of a marker gene product eg. β-galactosidase; or by selection for a dominant marker eg. HSV TK or neomycin resistance, concurrently inserted into the virus genome (28). The proportion of the progeny virus as recombinants can be increased by alternative transfection protocols involving the use of temperature sensitive mutants (5) or single stranded recombinant DNA molecules (34).

Many of these approaches rely on the TK phenotypes selection requiring TK^- cell lines. They are therefore not adaptable to other poxviruses such as fowlpox virus for which suitable TK^- cell lines are unavailable. Although TK^- recombinants are selectable under BUdR, the mutagenic effect of BUdR and the resulting background of TK^- mutants does not allow selection at the recombination step or enrichment at subsequent passage. The neomycin resistance gene can be used as a dominant selectable marker, however, high concentrations of G418 antibiotic are required with 48hr pretreatment for the selection to operate (35).

The herpes simplex virus TK (HSV-TK) gene has been inserted into non-essential regions in the vaccinia virus genome by positive selection protocols. By placing another gene under the control of a vaccinia virus promoter in tandem with the HSV-TK gene, positive selection protocols for the construction of recombinant vaccinia viruses expressing other genes have been achieved. This approach, however, requires the availability of a TK^- cell line which will survive for sufficient

time under the methotrexate (TK^+) selective conditions to allow growth and plaquing of TK^+ vaccinia viruses.

It has now been discovered that the use of the Escherichia coli xanthine-guanine phosphoribosyl transferase (Ecogpt) gene in novel combination with another gene, offers a route to recombinant poxviruses which does not suffer the aforesaid disadvantages of the prior art. In particular, it has been found that the Ecogpt gene can be used as a dominant selectable marker for insertion into poxvirus such as vaccinia virus. The antibiotic mycophenolic acid which, together with the required presence of xanthine, hypoxanthine, aminopterine and thymidine, provides the basis for selection using the Ecogpt gene is a very potent inhibitor of poxvirus growth. Since the Ecogpt gene has been used as a dominant selectable maker in a variety of mammalian cells, this obviates the need for TK^- cell lines for the construction of recombinant poxviruses (36).

The Ecogpt gene has previously been expressed from Simian Virus 40 - pBR322 hybrid plasmid vectors, and has been used as a dominant selectable marker in mammalian cells. Selection is based on the ability of the Ecogpt gene to counteract the inhibitory effect of mycophenolic acid on the growth of mammalian cells. Since mycophenolic acid is also a very strong inhibitor of poxvirus growth, this provides a marker for the insertion of the Ecogpt gene into poxviruses, so that when the Ecogpt gene is coupled in tandem with another gene of interest, recombinant poxviruses can be constructed and positively selected for the presence of both genes. The Ecogpt gene thus provides a dominant

selectable marker for insertion into any non-essential region of the poxvirus genome.

Accordingly, in another aspect, this invention provides a method of constructing a recombinant virus such as a poxvirus, adenovirus, herpesvirus, or the like, which comprises inserting into the non-essential region of a virus genome, a foreign DNA sequence comprising the Ecogpt gene coupled with a second gene, and selecting the virus recombinants by their ability to reverse the inhibition of virus growth by mycophenolic acid.

Potentially the Ecogpt/mycophenolic acid system provides a dominant selectable marker for insertion into any non-essential region of the genome of any virus where Ecogpt selection operates in the cell type in which the virus will grow. Not only will this facilitate the construction of recombinant vaccinia viruses, but it will also provide a method for the construction of recombinant virus vaccines including poxvirus vaccines based on host-specific poxviruses such as fowlpox virus for poultry or Orf virus for sheep, which are desirable because of the risk of disease spreading back to man from the use of vaccinia virus recombinants in animals.

In this aspect, therefore, the present invention also provides recombinant virus such as a poxvirus, adenovirus, herpesvirus or the like, characterised in that it has a foreign DNA sequence comprising the Ecogpt gene coupled with a second gene inserted into a non-essential region of the virus genome.

The second gene included within the foreign DNA sequence is preferably a gene which is expressed as a antigenic polypeptide which is foreign to the virus.

Operation of the Ecogpt gene as a dominant selectable marker for construction of recombinant poxviruses offers the additional advantages of allowing selective enrichment of recombinant viruses prior to plaque purification and characterization. It is also important in that it obviates the need for TK^- cell lines in the construction of recombinant viruses. This is of particular value in the construction of recombinant fowlpox viruses, since TK^- avian cell lines are not currently available and so it is not possible to adopt the general method for insertion of foreign DNA into vaccinia virus, which is based on the selective inactivation of the TK gene and the use of BUdR to select TK^- recombinants. Furthermore, use of the Ecogpt gene as a marker operates in most if not all eukaryotic cell types. The ability of confluent monolayers to maintain for extended periods and plaque poxviruses in the presence of MXHAT make it useful for the construction of recombinants based on slow growing host specific poxviruses. Further the ability to select at both the recombination and subsequent steps allows the construction of recombinants where the frequencies of recombination may be low e.g. the insertion of very large fragments of foreign DNA.

Those skilled in the art will be able to adapt known virus recombination techniques for insertion of specific Ecogpt/antigen gene couples into poxviruses. Essentially, the procedures involve insertion of a DNA sequence comprising the Ecogpt gene under the control of a poxvirus promoter plus a second gene under the control of the same or another poxvirus promoter, into a non-essential region of a

poxvirus genome. More than one promoter plus second gene may be coupled with the Ecogpt gene, thus enabling insertion into poxvirus of multiple antigens or multiple serotypes of one antigen.

Plasmids constructed with a second gene in tandem with the Ecogpt gene having flanking poxvirus sequences from non-essential regions could be used in the marker rescue recombination protocol. Recombinant virus containing the Ecogpt and second gene could be amplified and plaque-purified under mycophenolic acid selective conditions; all recombinants containing the Ecogpt gene would also contain the second gene, and this would be expressed provided it had been placed under the control of a poxvirus promoter. In the case of vaccinia and fowlpox virus, suitable promoters include P7.5, PF or PL 11.

Expression of the Ecogpt gene product is required for the recombinant viruses to grow under the mycophenolic acid selective conditions. The Ecogpt enzyme activity in virus-infected cell extracts can be demonstrated and measured using the conversion of ^{14}C-xanthine to ^{14}C-xanthine monophosphate and ^{14}C-xanthosine as described by Chu and Berg (24). Expression of the second gene inserted in tandem with the Ecogpt gene can be demonstrated by immuno chemical techniques, e.g. antibody and ^{125}I protein A binding to plaques for a cell surface - expressed antigen like the influenza HA gene.

In order to demonstrate that the Ecogpt gene and second gene have been inserted into the poxvirus genome in the correct position, DNA can be purified by known techniques from the recombinant viruses.

The purified DNA may then be analysed by restriction enzyme digests and DNA:DNA hybridisations to demonstrate the place and orientation in which the Ecogpt gene and second gene have been inserted.

The effectiveness of the recombinant viruses of this invention containing one or more foreign antigen genes as vaccines can be demonstrated by antibody and cell-mediated immume responses in animals infected with the recombinant viruses. Protection against disease can be demonstrated by challenge with the organism(s) from which the antigen gene(s) were derived.

Further features of the present invention will be apparent from the detailed description in the Examples hereinafter and in the accompanying drawings.

EXAMPLE 1

This Example illustrates the construction of recombinant FPV wherein the TK gene of the fowlpox virus is interrupted by the Ecogpt gene under control of a poxvirus promoter in tandem with a gene of interest under the control of another poxvirus promoter. By simultaneous infection of cells with FPV and transfection with recombinant plasmid in which the FPV TK gene was interrupted by the appropriate foreign genes and promoters, recombination occurs between the FPV TK flanking sequences of the plasmid and homologous sequences in the FPV genome. Selection of viruses expressing the Ecogpt gene simultaneously selects recombinants carrying both the Ecogpt gene and the gene of interest.

In Figures 1 to 8:

Figure 1 shows construction of a plasmid,

pDB16, containing multiple unique restriction enzyme sites within the TK gene of FPV. A HindIII-ClaI fragment of the FPV genome containing the TK gene was cloned into pUC9 which had been digested with EcoRI, mung bean nuclease treated and then digested with HindIII. The HindIII site was then deleted by HindIII digestion, Klenow PolI fill in and religation. A synthetic oligonucleotide was inserted into the unique NcoI site within the TK gene (pDB16). Unique sites for H, HindIII, C, ClaI, E, EcoRI and S, SmaI were inserted with this polylinker. Other restriction enzyme sites are N, NcoI and X, XbaI.

Figure 2 shows construction of plasmids for insertion of the Ecogpt and influenza genes into the TK gene of FPV. The Ecogpt gene with attached P7.5 vaccinia virus promoter (as an EcoRI-AhaIII fragment) was subcloned from pGpt07/14 into pDB16 which had been digested with EcoRI and SmaI (pDB18). Then the HA gene under the control of the PL11 vaccinia virus promoter was cloned into pDB18 as a ClaI fragment from pBCB08/HA. Plasmids pDB19/1 and pDB19/6 have the FPV TK gene interrupted by the P7.5-Ecogpt and PL11-HA gene fragments. Abbreviations for restriction enzyme sites; C, ClaI, E, EcoRI, H, HindIII, N, NcoI, X, XbaI. CIAP, calf intestinal alkaline phosphatase.

Figure 3 shows construction of insertion vectors for the selection of FPV recombinants using the Ecogpt gene and having foreign genes under the control of the PL11 vaccinia virus promoter. In pDB20 the size of the flanking FPV genome sequences has been increased by subcloning the P7.5-Ecogpt-PL11-HA fragment into the 5.5 EcoRI FPV genome

fragment at the unique NcoI site contained in pDB1. pDB22 is a general vector into which foreign DNA can be inserted downstream of the PL11 vaccinia virus promoter and recombinants selected on the basis of growth in Ecogpt selective conditions. Abbreviations for restriction enzyme sites; B, BamHI; C, ClaI; E, EcoRI; H, HindIII; N, NcoI; P, PstI; S, SmaI and X, XbaI.

Figure 4 shows antibody and ^{125}I protein A binding to FPV plaques. A, normal mouse serum; B, hyperimmune mouse antiserum to influenza virus A/PR/8/34. FPV, fowlpox virus; FPV-HA, fowlpox virus recombinant containing the Ecogpt-P7.5 promoter and the influenza HA-PL11 promoter constructed using pDB20.

Figure 5 shows the structure of the TK region of the parent, FPV-M3, and the recombinant, FPV-HA, genomes. The recombinant, FPV-HA, was constructed using pDB20 (Fig.4). Abbreviations for restriction enzyme sites: E, EcoRI; N, NcoI; P, PstI; S, SalI. Not all EcoRI and NcoI sites are shown.

Figure 6 shows analysis of DNAs from the parent, FPV-M3 (1) and the recombinant FPV-HA (2) viruses. DNAs were digested with SalI (S) and PstI (P) and separated by electrophoresis through 0.6% agarose gels containing 0.5µg/ml ethidium bromide (EtBr). The gel was photographed with UV illumination then blotted onto Gene-Screen Plus. The membrane was then hybridized with various ^{32}P labelled DNA fragments (pDB1; HA; Ecogpt; P7.5; PL11).

Figure 7 shows the HI antibody response of chickens inoculated with FPV of FPV-HA (5

birds/group), 15 day old SPV chickens were inoculated with 10^6 to 10^7 pfu of virus and reinoculated 21 days later. For birds inoculated with FPV-HA titers for individual birds are shown by the dots. The geometric mean titer for these birds is shown by the stars. For the birds inoculated with the vaccine virus or FPV-M3 the titers were never greater than 10 and are shown by the solid squares.

Figure 8 shows the location, nucleotide sequence, and derived amino acid sequence of the FPV TK gene and flanking regions. The nucleotide sequence was determined as described by Boyle and Coupar (14) and Boyle et.al. (15). Restriction enzyme cleavage sites are indicated as follows: E, EcoRI; H, HindIII; X, XbaI; C, ClaI. Not all HindIII, XbaI, and ClaI sites present in the 5,5-kb EcoRI fragment are shown. The position of the FPV TK gene was confirmed by its homology to the vaccinia virus TK gene. The derived amino acid sequence is presented using the single-letter amino acid code. The Sau96I-DraI fragment used as primer for 5' end mapping of mRNA is indicated by the line above the squence. The initiation and termination codons of the second open reading frame identified in this fragment are similarly indicated. * Termination codon.

MATERIALS AND METHODS

Enzymes and Plasmids.

Restriction endonucleases and DNA-modifying enzymes were obtained from several commercial suppliers and used according to the manufacturers' instructions or those described in detail by Maniatis et.al. (17). Recombinant plasmids were prepared from the pUC (25) series and DNA purified in $CsCl_2$

gradients. pDB1 to pDB11 have been described previously (14). pBCB08, a multiple cloning site plasmid for the construction of recombinant vaccinia viruses, contains the late vaccinia virus promoter PL11 (26,30). The A/PR/8/34 influenza HA gene in pJZ102 was provided by Dr.P.N.Graves, The Mount Sinai Medical Center (27). The P7.5 vaccinia virus promoter in pGS20 was provided by Dr.B.Moss, National Institutes of Allergy and Infectious Diseases, Bethesda, Md. (2).

Cell cultures and viruses.

Primary and secondary chick embryo skin (CES) cell cultures were prepared from specific pathogen free embryonated eggs (CSIRO, SPF Poultry Unit, Maribyrnong, Victoria) as described by Silim et.al. (18) with the modification that collagenase at 100µg/ml (Sigma C2139) was used to digest the skin of the embryos in place of trypsin. Fowlpox virus. (Mild Vaccine Strain: Arthur Webster Pty.Ltd., Northmead, NSW, 2152, Australia) was adapted to chick embryo cell cultures by passage at low multiplicity and plaque purified twice. This virus, designated FPV-M3, contains an approximately 10kb deletion from its genome. Plaque assays were performed on CES monolayers overlayed with Eagle's minimum essential medium with Earle's salts containing 1% agar and 5% foetal bovine serum. Plaques were stained with MTT tetrazolium (19) on the fifth or sixth day after inoculation. All virus stocks were disaggregated by digestion for 30min at 37°C with 1mg/ml trypsin immediately prior to dilution for assays or infection of cell cultures.

Marker Rescue.

CES cells seeded overnight at $5x10^6$

cells/25cm^2 flask were infected with FPV-M3 at 0.01 plaque forming units per cell. Virus was adsorbed for 1hr at 37°C then culture medium added. 7hr later the monolayers were washed four times with medium without serum and buffered with 0.05M Tris pH7.4, then once with Tris buffered saline (25mM Tris pH7.4, 137mM NaCl, 5mM KCl, 0.7mM $CaCl_2$, 0.5mM $MgCl_2$, 0.6mM Na_2HPO_4; TBS; (31). To each flask was added 2ml medium without serum containing 20µg of plasmid in DEAE-Dextran (MW=2x10^6, Pharmacia) at a final concentration of 200µg/ml. The DNA solution was prepared by dissolving 20µg of plasmid (purified by twice banding in $CsCl_2$ gradients) in 125µl of TBS, adding 40µl of DEAE-Dextran at 10mg/ml and then adding 2ml of culture medium without serum (31). The cultures were then incubated for a further 16hr at 37°C at which time they were washed twice with TBS. Medium with 5% foetal bovine serum and selective conditions were then added (MXHAT= 1 or 2·5µg/ml mycophenolic acid, 250µg/ml xanthine, 100µM hypoxanthine, 0.4µM aminopterine and 30µM thymidine).

The cultures were incubated for 6 or 7 days at which time the cells were scraped into the medium, pelleted by centrifugation and resuspended in 1ml of phosphate buffered saline. This virus stock was then passaged two or three times on CES cells under selective conditions. At the second and subsequent passages plaques grew out from marker rescues performed with plasmids containing the Ecogpt gene. Recombinant viruses containing the Ecogpt gene were plaque purified under agar without selection then amplified under selection in liquid medium in 24 well plates. Viruses containing the Ecogpt gene and gene

of interest were identified by dot blot hybridization with ^{32}P-labelled recombinant DNA probes (2,3).

Characterisation of recombinant FPVs.

FPV was purified from CES cultures (21) and the DNA extracted as described by Nakano et.al. (22). DNA was subjected to restriction enzyme digestion and separated by agarose gel electrophoresis. After blotting to Gene-Screen Plus membrane (NEN), ^{32}P-labelled DNA probes were hybridized to the membrane to analyse the arrangement of the virus genome.

Expression of influenza haemagglutinin by recombinant viruses was demonstrated by binding of anti-influenza antibodies and ^{125}I-protein A to FPV plaques. The solid overlay was removed from plaques then the monolayer fixed for 5mins at room temperature with methanol. Antibodies and ^{125}I-protein A were then bound to the plaques as previously described (1,11).

Responses of Poultry to recombinant FPVs.

SPF poultry, 15 days old, were inoculated subcutaneously/intradermally (s.c./i.d.) into the breast skin with 0.05ml of virus containing 10^6-10^7 pfu. Serum was collected at 7 day intervals. The birds were reinoculated s.c./i.d. into the wing web 21 days later. Sera were tested against 4 haemagglutinating units (HAU) of A/PR/8/34 virus. Serum dilutions were two-fold beginning at 1:5. End points were recorded as the dilution giving complete inhibition of 4HAU and the titre was expressed as the reciprocal of that dilution.

Construction of insertion vectors.

The FPV TK gene is contained within a 2.2kb

HindIII-ClaI fragment of the FPV genome (14). This fragment was subcloned from pDB10 (14) between the HindIII-EcoRI sites of pUC9 (Fig.1; pDB14). The remaining HindIII site was deleted by digestion, fill in with Klenow fragment of polymerase I and religation (pDB15). Inspection of the TK gene nucleotide sequence revealed a unique NcoI site located in the centre of the TK gene and about 20bp away from the XbaI site also in this gene (15). Restriction enzyme analysis revealed that the NcoI site was unique to the 5.5kb EcoRI fragment of the FPV genome originally shown to contain the TK gene (15).

To facilitate the insertion of foreign DNA sequences within the TK gene, a synthetic oligonucleotide linker with NcoI cohesive ends and restriction enzyme sites for SmaI, EcoRI, ClaI and HindIII was inserted into this unique NcoI site (pDB16). The orientation of the linker with respect to the TK gene was determined by restriction enzyme digests and polyacrylamide gel electrophoresis. pDB16 was then used as the backbone for insertion of foreign sequences within the FPV TK gene.

The Ecogpt gene from pSV2A-gpt has previously been cloned into pBCB07 (29). A 900bp fragment, derived by EcoRI-AhaIII digestion, containing the Ecogpt gene with the vaccinia virus promoter P7.5 attached was cloned into pDB16 digested with EcoRI-SmaI. The resulting plasmid pDB18 contains the TK gene interrupted by the Ecogpt gene under the control of the P7.5 promoter.

The influenza A/PR/8/34 haemagglutinin gene had been inserted previously into pBCB08 at the HindIII site of the multiple cloning site (30). A

ClaI fragment from pBCB08/HA containing the PL11 promoter and HA gene was subcloned into pDB18 which had been digested with ClaI and treated with calf intestinal alkaline phosphatase. This placed the influenza HA gene under the control of the PL11 promoter in tandem with the Ecogpt gene all contained within the FPV TK gene (pDB19/1 and pDB19/6) (Fig.2).

In an attempt to increase the frequency of recombination by having larger flanking sequences, the Ecogpt in tandem with the HA gene was subcloned from pDB19/6 into the unique NcoI site of pDB1 (Fig.3; pDB20). pDB1 contains a 5.5kb EcoRI fragment of the FPV genome in which the TK gene is contained.

To construct a general insertion vector, the HA gene in pDB19/1 was removed by HindIII digestion and religation. pDB22 contains the FPV TK gene interrupted by the P7.5 promoter-Ecogpt gene plus the PL11 promoter. Attached to the PL11 promoter is a multiple cloning site with unique restriction enzyme sites for SmaI, BamHI, SalI, PstI and HindIII (Fig.3). This vector is suitable for the insertion of a variety of genes into the FPV genome using the Ecogpt gene to select for the recombinants.

Construction and characterization of FPV recombinants expressing influenza haemagglutinin.

Preliminary experiments showed that FPV plaquing and growth in CES cells was strongly inhibited by mycophenolic acid using the MXHAT selective conditions. This was similar to the inhibition of vaccinia virus by MXHAT (29).

Insertion of the influenza HA gene into FPV in tandem with the Ecogpt gene was achieved using pDB20. Virus plaques which grew in the presence of

MXHAT were plaque purified three times and confirmed as containing the Ecogpt and HA genes by dot blot hybridization. Subsequently recombinant FPVs were successfully constructed using pDB18 and pDB19 which have shorter FPV flanking sequences.

Expression of influenza haemagglutinin by the recombinant FPV-HA was demonstrated by the binding of antibodies and ^{125}I-protein A to plaques. Only plaques of FPV-HA bound antibodies to influenza virus whilst the wild type virus, FPV-M3, failed to do so. Neither virus bound antibodies from normal serum (Fig.4).

The structure of the recombinant, FPV-HA, genome was analysed by restriction enzyme digestion and Southern hybridization in comparison with the parent virus, FPV-M3 (Figs. 5 and 6). The 5.5kb EcoRI fragment which contains the TK gene spans the junction of two PstI fragments of 26kb and 13kb (Fig.5). The TK gene is contained wholly within the 26kb fragment. PstI digestion of the FPV-M3 genome showed in ethidium bromide stained gels that the 26kb fragment was replaced by 23 and 6kb fragments. With SalI digestion a new 15.5kb fragment was present (Figs. 5 and 6).

The agarose gel separated fragments were transferred to Gene-Screen-Plus membrane (NEN) and successively hybridized with various ^{32}P-labelled DNA fragments (Fig.6). After each hybridization and autoradiography the ^{32}P-labelled probe was removed (as per manufacturers instructions) and the membrane hybridized with a different ^{32}P-labelled fragment. By insertion of the foreign DNA in pDB20 into the FPV genome an additional PstI site within the PL11 multiple cloning site would be present.

Hybridization with the EcoRI, 5.5kb, fragment in pDB1 showed that wild type pattern of 26 and 13kb fragments changed to new fragments of 23 and 6kb with the 13 kb fragment unchanged. The SalI pattern was also changed with a large unresolved fragment replaced by a smaller fragment and the 15.5kb fragment present in the ethidium bromide stained gel (Fig.5). A SalI site had been inserted as part of the multiple cloning site of the PL11 promoter.

When hybridized with the HA or Ecogpt genes or the P7.5 promoter, the patterns were the same. As expected all hybridized with the new 6kb PstI fragment and the large SalI fragment. None hybridized with the genome of the parent virus, FPV-M3. As predicted by the restriction enzyme map in Fig.5 the PL11 promoter hybridized with the 15.5kb SalI and the 23kb PstI fragments.

The ethidium bromide stained gel and the hybridization patterns confirmed that all the foreign DNA elements in pDB20 were inserted into the FPV genome in the expected position and orientation. Other restriction enzyme digests and hybridization analyses with FPV-M3 DNA using BamHI,EcoRI and HindIII were also as expected.

Response of poultry to recombinant FPV's

SPF chickens inoculated at 16 days of age with the FPV mild vaccina strain or FPV-M3 failed to develop haemagglutination inhibiting antibodies (HI) to the A/PR/8/34 influenza virus during the next 43 days. All birds developed a FPV lesion at the primary inoculation site. No lesions developed at the secondary stage inoculation site.

Chickens inoculated with the recombinant FPV-HA, developed a lesion at the inoculation site

and HI antibodies to the influenza virus A/PR/8/34. Antibodies were detected on day 9 after inoculation and the titers increased thereafter. Maximum titers of 40 to 320 occured 29 days after inoculation and they remained stable till day 43. Following reinoculation at day 21 there was not a marked secondary antibody response, however, titers prior to this time were continuing to rise and consequently any secondary response may have been masked by the rising antibody response (Fig. 7).

EXAMPLE 2

This Example demonstrates that insertion of the Ecogpt gene into vaccinia virus under the control of a vaccinia virus promoter leads to vaccinia virus recombinants able to grow in the presence of MXHAT. When coupled in tandem with another gene of interest vaccinia virus recombinants can be constructed and positively selected carrying both genes.

In Figures 9 to 11:

Figure 9 shows plaquing of vaccinia virus on CV-1 monolayers in the presence of MXHAT (1 or 25 µg/ml of mycophenolic acid). 1, 2 and 3, VV-WR; 4, VV-Ecogpt; 3, guanine at 25 µg/ml was able to reverse the inhibition of plaque formation by MXHAT.

Figure 10 shows construction of plasmids for the insertion of the Ecogpt gene into the TK gene of vaccinia virus. H, HindIII; B, BamHI; E, EcoRI; MCS, multiple cloning site; CIAP, calf intestinal alkaline phosphatase.

Figure 11 shows the effect of selection and moi at the recombination step on the output of recombinant viruses expressing the Ecogpt gene. Monolayer cultures of CV-1 cells (10^6cell/25cm^2

flask) were infected with VV-WR at various moi's. Two hours later the cultures were transfected with $CaPO_4$ precipitated plasmid DNA (2µg of pGpt07/14 and 20µg of calf thymus DNA). Eight hours later the medium was changed, with or without the addition of MXHAT selection. Six days later the cells were harvested and assayed on CV-1 monolayers in the presence or absence of MXHAT. The % of the output as recombinants is plotted on a $\log_{10}$ scale.

no selection applied at recombination step
selection applied at recombination step.

EXPERIMENTAL

(a) Inhibition of vaccinia virus plaquing by mycophenolic acid.

Mycophenolic acid has previously been used at 25µg/ml to inhibit mammalian cell growth and to select for cells carrying the Ecogpt gene (36,37). Preliminary experiments established that confluent monolayers of CV-1 cells used for the vaccinia virus plaque assay survived for more than 7 days in mycophenolic acid up to 25µg/ml. When mycophenolic acid (MXHAT) was tested against vaccinia virus on preformed monolayers of CV-1 cells, doses from 1 to 25µg/ml completely inhibited plaque formation and even at 0.1µg/ml there was significant reduction in plaque size (Fig. 9). Inhibition of mammalian cell growth by mycophenolic acid is reversible by guanine. Addition of guanine, 25 µg/ml, to plaque assays in the presence of MXHAT (M from 0.1 to 25µg/ml) reversed the inhibition of plaque formation and plaque sizes approached normal (Fig. 9).

This showed that the mycophenolic acid was inhibiting vaccinia virus growth by acting as an

inhibitor of inosine monophosphate dehydrogenase preventing the formation of xanthine monophosphate and therefore guanine monophosphate in a manner similar to its action on mammalian cells (37). Mycophenolic acid alone failed to inhibit vaccinia virus plaque formation on CV-1 cells. HAT (hypoxanthine, aminopterine and thymidine) was required to inhibit de novo synthesis of inosine monophosphate to allow expression of the mycophenolic acid inhibition. HAT alone had no effect on vaccinia virus plaquing on CV-1 cells. The conditions used to inhibit completely vaccinia virus plaquing even at very high virus input were mycophenolic acid, 1 or 2.5μg/ml, xanthine, 250μg/ml, hypoxanthine, 100μM, aminopterine, 0.4μM, thymidine, 30μM (MXHAT). The cell monolayers do not require pretreatment with MXHAT for inhibition of virus plaquing and preformed monolayers maintain for long periods (7 to 10 days) under MXHAT selection.

(b) Insertion of the Ecogpt gene into vaccinia virus

The Ecogpt gene and attached SV40 polyadenylation signals from pSV2A-gpt (36) was subcloned as a HindIII-BamHI fragment into pUC9. The translation initiation codon of the Ecogpt gene is located 200bp from the HindIII site. A unique BglII site is located 120bp closer to the translation initiation codon (38). To position the Ecogpt gene as close to the vaccinia virus promoter as possible, a BglII-BamHI fragment was cloned into pBCB07 (39; Fig.10). Recombinant plasmids pGpt07/14 and 15 were selected by restriction enzyme digests as having the Ecogpt gene in the correct and incorrect orientation with respect to the vaccinia virus promoter and

flanked by the TK gene sequences.

Only the plasmid pGpt07/14 having the Ecogpt gene in the correct orientation with respect to the vaccinia virus promoter was able to generate recombinant viruses which could grow in MXHAT. In a typical experiment, 0.1 to 0.2% of the output virus from the recombination step where selection was not applied at this step were recombinants. A single passage at low moi (0.01) from the original recombination step resulted in 10 to 30% of the output virus being recombinants. When MXHAT selection was applied at the recombination step, the proportion of recombinants in the output increased dramatically. At a moi of 1 the proportion of recombinants increased from 0.1% to 0.5%. When low moi's (0.01 and 0.001) were used and the incubation in MXHAT extended to 5 to 7 days for the first step, recombinants represented 10 to 40% of the output compared with the normal 0.1 to 0.2 (Fig.11; the total output of virus was reduced by 100 to 1000 fold).

The ability to positively select at the recombination and subsequent steps plus the absence of spontaneous background mutants greatly facilitates the construction and selection of recombinants. A recombinant vaccinia virus has been constructed using pGpt07/14 and the insertion of the Ecogpt gene into the TK region confirmed by restriction enzyme analysis and Southern hybridization analysis. This recombinant, VV-Ecogpt, is able to plaque under MXHAT on CV-1 monolayers whilst plaquing of the wild type virus, VV-WR, is completely inhibited (Fig. 9). Large fragments of fowlpox virus genome, up to 20kb, have been inserted into vaccinia virus using the

pGpt07/14 vector as a selectable insertion vehicle. Additional genes of interest could be inserted and expressed in vaccinia virus by inserting another promoter plus gene into pGpt07 then using the Ecogpt gene to select for recombinants.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications which fall within its spirit and scope.

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CLAIMS:

1. Recombinant fowlpox virus or related avian poxvirus, characterised by the inclusion of foreign DNA in the virus genome.

2. Recombinant virus according to claim 1, wherein said foreign DNA sequence is inserted in a non-essential region of the virus genome.

3. Recombinant virus according to claim 2, wherein said foreign DNA is inserted in the TK gene of the virus or in virus DNA sequences controlling expression of the TK gene.

4. Recombinant virus according to claim 1, wherein said foreign DNA comprises a DNA sequence encoding an antigenic polypeptide.

5. Recombinant virus according to claim 4, wherein said foreign DNA comprises a DNA sequence encoding an antigen characteristic of an avian disease.

6. Recombinant virus according to claim 4 or claim 5 wherein said foreign DNA comprises a DNA sequence encoding more than one antigenic polypeptide.

7. Recombinant fowlpox virus for use as an avian disease vaccine characterised by the inclusion of a foreign DNA sequence encoding an antigen characteristic of the said avian disease, said foreign DNA sequence being inserted in the region of the fowlpox virus commencing 279bp before and

terminating 273bp after the central XbaI site of the nucleotide sequence shown in Fig.8, or in sequences controlling expression of this sequence.

8. Recombinant virus, according to any one of claims 1 to 7, wherein said foreign DNA includes the Escherichia coli xanthine-guanine phosphoribosyl transferase gene (Ecogpt), coupled with one or more additional foreign genes.

9. Recombinant virus according to any one of claims 1 to 8, wherein said foreign DNA is under control of at least one poxvirus promoter.

10. Recombinant virus according to claim 9, wherein the or each poxvirus promoter is a fowlpox virus or related avian poxvirus or vaccinia virus promoter.

11. Recombinant virus according to claim 10, wherein the or each poxvirus promoter is selected from the vaccinia virus promoter sequences P7.5 and PL11.

12. Recombinant virus according to claim 8, wherein said foreign DNA includes the Ecogpt gene under control of the vaccinia virus P7.5 promoter, coupled with one or more additional foreign genes under control of the same or additional poxvirus promoters.

13. A method for the construction of recombinant fowlpox virus or related avian poxvirus, which comprises the introduction of foreign DNA into the TK

gene of the virus or into virus DNA sequences controlling expression of the TK gene.

14. A method for inducing immunity in poultry to an avian disease, which comprises administering to said poultry an avian disease vaccine comprising recombinant fowlpox virus or related avian poxvirus characterised by inclusion of a foreign DNA sequence encoding an antigen characteristic of the said avian disease in the TK gene of the virus or in virus DNA sequences controlling expression of the TK gene.

15. A fowlpox or related avian poxvirus vaccine which comprises TK^- fowlpox virus or related avian poxvirus.

16. Recombinant virus characterised in that it has a foreign DNA sequence comprising the Escherichia coli xanthine-guanine phosphoribosyl transferase gene (Ecogpt) coupled with a second gene inserted into a non-essential region of the virus genome.

17. Recombinant virus according to claim 16, wherein the virus is a poxvirus.

18. Recombinant poxvirus according to claim 14 or 15, wherein the poxvirus is vaccinia virus, fowlpox virus or Orf virus.

19. Recombinant virus according to claim 16, wherein the foreign DNA sequence is inserted into the TK gene of the virus.

20. Recombinant virus according to any one of

claims 16 to 19, wherein said second gene is expressed as an antigenic polypeptide which is foreign to the virus.

21. Recombinant virus according to any one of claims 16 to 20, wherein in said foreign DNA sequence said Ecogpt gene is under control of a poxvirus promoter and said second gene is under control of the same or another poxvirus promoter.

22. Recombinant virus according to any one of claims 16 to 21, wherein said foreign DNA sequence contains one or more additional genes.

23. A method of constructing a recombinant virus which comprises inserting into a non-essential region of the virus genome, a foreign DNA sequence comprising the Ecogpt gene coupled with a second gene, and selecting the virus recombinants by their ability to reverse the inhibition of virus growth by mycophenolic acid.

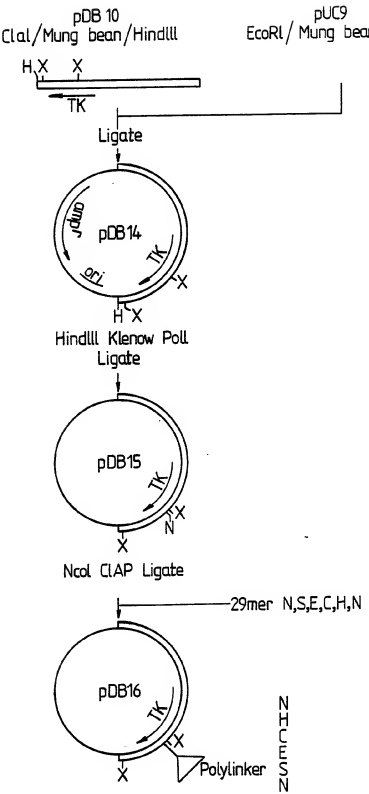

FIG. 1.

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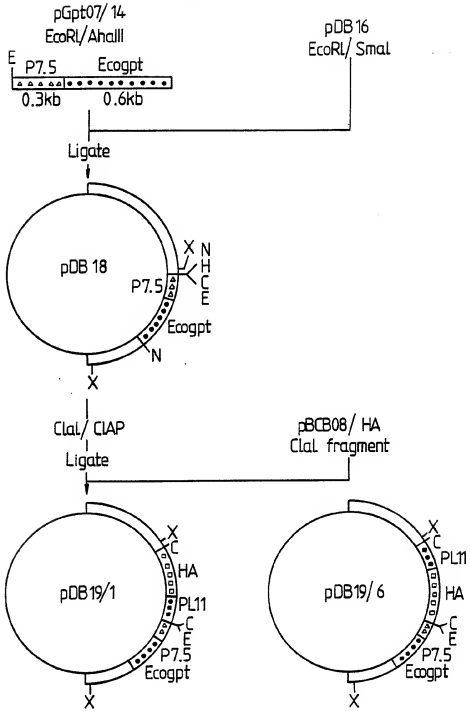
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P7.5
Ecogpt
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H
C
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N
X
ClaI/ CIAP
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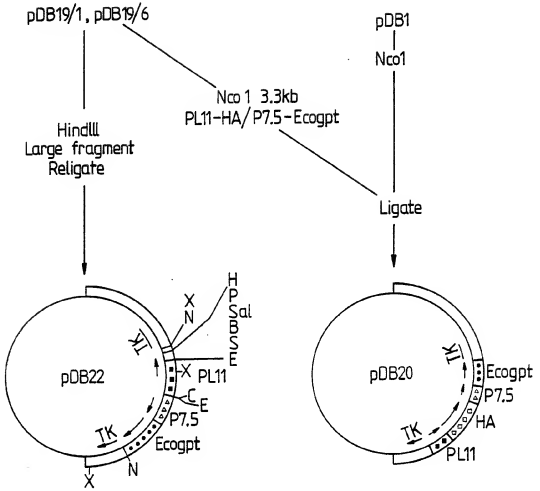
FIG. 2.

3/13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HindIII
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Religate
Nco 1 3.3kb
PL11-HA/P7.5-Ecogpt
pDB1
Nco1
Ligate
pDB22
TK
X
N
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FIG.3.

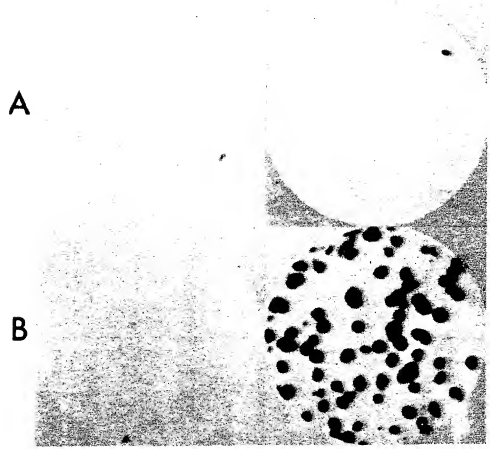
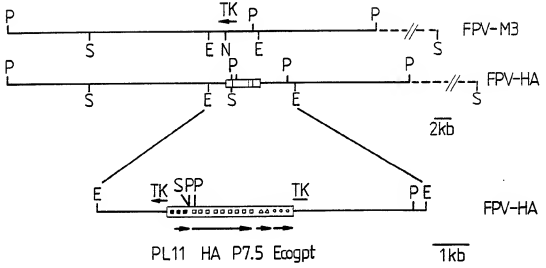


FIG. 4.

5/13



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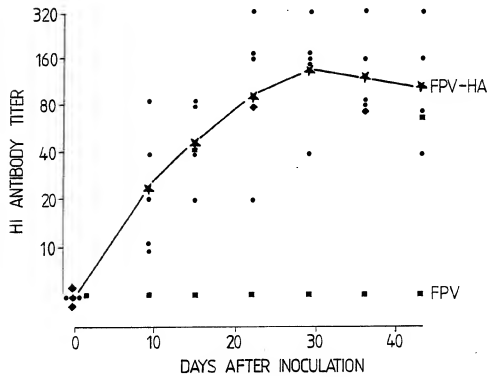
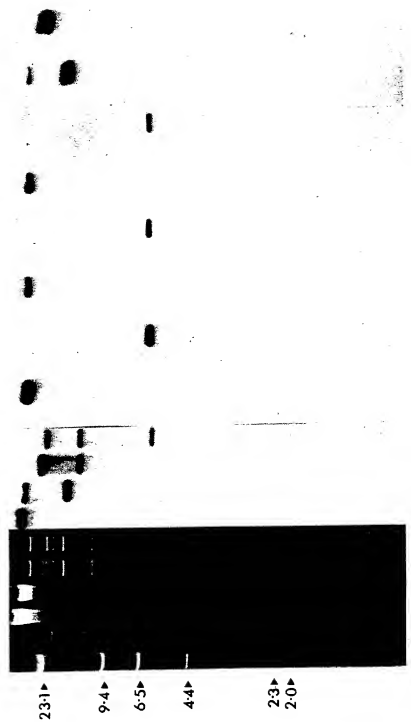


FIG. 7.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6·5
4·4
2·3
2·0
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pDB1
HA
Ecogpt
P7·5
PL11

FIG.6.

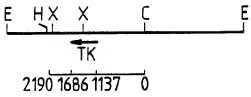


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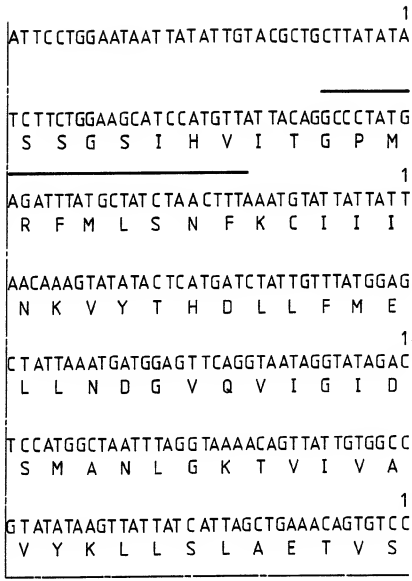
Fig.8(a).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N K V Y T H D L L F M E
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Fig.8(b).

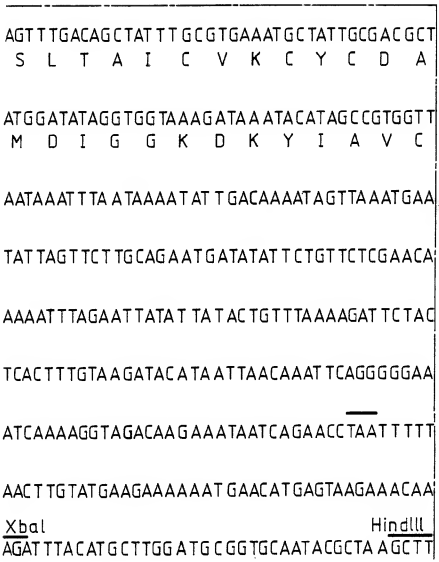

Fig.8(c).

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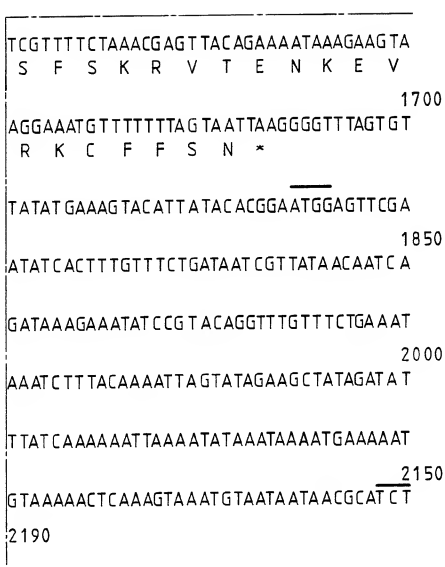
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R K C F F S N *
TATATGAAAGTACATTATACACGGAATGGAGTTCGA
1850
ATATCACTTTGTTTCTGATAATCGTTATAACAATCA
GATAAAGAAATATCCGTACAGGTTTGTTTCTGAAAT
2000
AAATCTTTACAAAATTAGTATAGAAGCTATAGATAT
TTATCAAAAAATTAAAATATAAATAAAATGAAAAAT
2150
GTAAAAACTCAAAGTAAATGTAATAATAACGCATCT
2190

Fig.8(d).

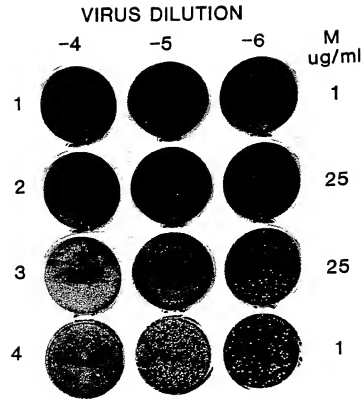

FIG.9.

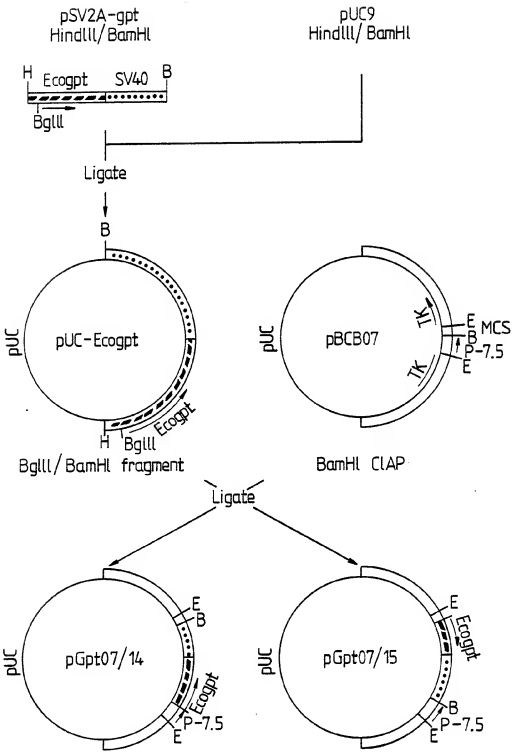
pSV2A-gpt
HindIII/ BamHI
pUC9
HindIII/ BamHI
H
Ecogpt
SV40
B
BglII
Ligate
B
pUC
pUC-Ecogpt
H
BglII
Ecogpt
BglII/ BamHI fragment
pUC
pBCB07
TK
E
MCS
B
P-7.5
E
TK
BamHI CIAP
Ligate
pUC
pGpt07/14
E
B
Ecogpt
P-7.5
E
pUC
pGpt07/15
E
Ecogpt
B
P-7.5
E

FIG. 10.

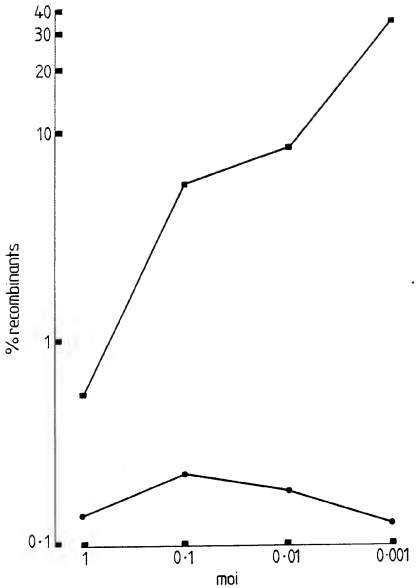

FIG. 11.

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 87/00323

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C12N 15/00, 7/00, A61K 39/275, 39/285 // (C12N 15/00, C12R 1:91)

II. FIELDS SEARCHED

Minimum Documentation Searched [7]	
Classification System	Classification Symbols
IPC	WPI, WPIL Keywords : FOWLPOX, AVIANPOX, ECOGPT, XANTHANE GUANINE PHOSPORIBOSYL

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

AU : IPC C12N 15/00
BIOTECHNOLOGY, CHEMICAL ABSTRACTS KEYWORDS AS ABOVE

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category * [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X,Y	WO,A, 86/00528 (GENEX CORPORATION) 30 January 1986 (30.01.86) See pages 24-28	(1-6,9,10, 13-15)
X,Y,P	WO,A, 86/05806 (NATIONAL RESEARCH DEVELOPMENT CORPORATION) 9 October 1986 (09.10.86) See page 23	(1-6,13-15)
Y	Journal of General Virology, Volume 67, No.8, issued 1986 August (Colchester, England), D.B. Boyle et al 'Identification and Cloning of the Fowlpox Virus Thymidine Kinase Gene Using Vaccinia Virus', see pages 1591-1600	(1-6,13-15)
Y,P	Virology, Volume 156, no.2, issued 1986 January (New York, USA), D.B. Boyle et al 'Fowlpox Virus Thymidine Kinase: Nucleotide Sequence and Relationship to Other Thymidine Kinases', see pages 355-365	(1-6,13-15)
X	Proceedings of the National Academy of Sciences USA, Volume 81, no.22 issued 1984, November (Washington D.C. USA), H. Stuhlmann et al, 'Introduction of a selectable gene into different animal tissue by a retrovirus recombinant vector', see pages 7151-7155	(16)

(continued)

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 December 1987 (17.12.87)	(12.01.88) 12 JANUARY 1988
International Searching Authority: Australian Patent Office	Signature of Authorized Officer: J.W. ASHMAN

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	Molecular and Cellular Biology, Volume 3, No.8, issued 1983, August (Washington D.C. USA), A.B. Chapman et al, 'Amplification and Hormone-Regulated Expression of a Mouse Mammary Tumour Virus - Ecogpt Fusion Plasmid in Mouse 3T6 Cells', see pages 1421-1429	(16)
X	Experimental Cell Research, Volume 166, no.1, issued 1986, January (New York, USA), M. Mevel-Ninio et al, 'A Polyoma-Derived Plasmid Vector Maintained Episomally in Both E. Coli and Mouse Hepatoma Cells', see pages 63-76	(16)

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . . . . , because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers . . . . . , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically

3. ☐ Claim numbers..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 87/00323

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members
WO 8600528	EP 190254
WO 8605806	EP 218625 GB 8508265

END OF ANNEX